Sex-specific differences in immunological costs of multiple mating in *Gryllus vocalis* field crickets

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Although mating can be costly, most individuals must choose not whether to mate but how many times to mate. This study examined the immunological costs of additional matings once an individual has already mated. *Gryllus vocalis* field crickets were assigned to mate 5 or 10 times and their immunocompetence probed by measuring their ability to withstand a bacterial challenge by *Serratia marcescens*, assaying lysozyme-like enzyme activity and phenoloxidase activity, and measuring their success in encapsulating a monofilament implant. Although number of matings generally did not affect the strength of immune responses, females had superior immunity to males in most assays. Females that mated 10 times did, however, have lower lysozyme-like enzyme levels than females that mated 5 times, suggesting that mating can compromise at least one component of female standing immunity. When individuals were allowed to mate ad libitum and their lysozyme-like enzyme activity, and encapsulation responses measured, there was not a relationship between female mating frequency and lysozyme-like enzyme activity. This result suggests that females may avoid immunological costs of mating by differentially moderating their mating frequency. **Key words:** encapsulation, *Gryllus vocalis*, insect immunity, life-history trade-offs, lysozyme, phenoloxidase, polyandry, *Serratia marcescens*. [Behav Ecol 19:810–815 (2008)]

**METHODS**
Vocal field crickets, *Gryllus vocalis* (Weissman et al. 1980), were collected from the Botanic Gardens at the University of California, Riverside. The laboratory colony was initiated with 50 adult males and 50 adult female crickets in spring 2002 and supplemented each subsequent spring with 30–100 additional field-caught adults. Crickets were maintained in a growth chamber under simulated summer conditions: 28 °C with a daily cycle of 14 h light to 10 h dark. Animals were given access to moistened cotton for water and oviposition and maintained on a diet of rabbit chow provided ad libitum.

**Insect immunity**

The insect immune system consists of humoral and cell-mediated components. Humoral responses include the production of antimicrobial compounds that defend against bacterial, viral, or fungal pathogens (Abbas and Lichtman 2003). Cell-mediated responses include the encapsulation or melanization of foreign objects within the body cavity by covering them with melanin and hemocytes. In this study, I examined both cell-mediated and humoral responses. First, I assessed the ability of individuals to survive exposure to the challenge by *Serratia marcescens*, assaying lysozyme-like enzyme activity and phenoloxidase (PO) activity, and measuring their success in encapsulating a monofilament implant. To determine whether individuals can avoid immunological costs by adjusting their mating rate, I conducted a second study in which crickets were allowed to mate ad libitum, and then measured their lysozyme-like enzyme activity and PO activity, and assessed their success in encapsulating an implant.

**RESULTS**

Here, I report the results of 2 studies designed to assess how additional matings affect male and female immunity and whether such matings affect male and female immunity differently. In the first study, I experimentally assigned males and females to mate 5 or 10 times and then probed their immunocompetence by measuring their ability to withstand a bacterial challenge by *Serratia marcescens*, assaying lysozyme-like enzyme activity and phenoloxidase (PO) activity, and measuring their success in encapsulating a monofilament implant. To determine whether individuals can avoid immunological costs by adjusting their mating rate, I conducted a second study in which crickets were allowed to mate ad libitum, and then measured their lysozyme-like enzyme activity and PO activity, and assessed their success in encapsulating an implant.
bacteria *S. marcescens*, which is commonly found in wild orthopterans (Bucher 1959) and has been demonstrated to decrease survival in the laboratory (Stevenson 1959; Adamo et al. 2001). Second, I estimated the amount of the inactive hemolymph-bound enzyme prophenoloxidase. PO catalyzes the reaction of dopamine into melanin, a key step in the encapsulation response pathway (Söderhäll and Cerenius 1998). Third, I estimated the amount of a hemolymph-bound enzyme lysozyme, which defends against bacteria. Finally, I measured the degree to which a foreign body was engulfed by hemocytes and melanized, providing a measure of macro-parasitic defense. Bacterial challenge and encapsulation ability are measured in vivo as the response of individuals to a novel immune challenge and thus represent a “realized” immune response. However, estimates of lysozyme and PO are “potential” responses because they measure in vitro the amount of enzyme present, providing information about the potential of an individual to mount an immune response. Bacterial challenge (Adamo et al. 2001; McKean and Nunney 2001), PO activity (Adamo et al. 2001; Siva-Jothy et al. 2001; Fedorka and Zuk 2005), lytic activity (Rantala and Kortet 2003), and implant encapsulation (Siva-Jothy et al. 2001; Rantala and Kortet 2003; Zuk et al. 2004) are commonly used to assess insect immunity.

**The effect of experimentally assigned matings on immune responses**

**Experiment Ia: Experimental infection with *S. marcescens***. I randomly assigned 200 virgin males and 194 virgin females to mate either 5 or 10 times. These mating frequencies correspond roughly to those observed in free-living crickets in nature (e.g., Sakuluk et al. 2002). In addition, previous experiments have demonstrated that female *Gryllus* males mate 10 times lay more eggs, and a higher proportion of fertilized eggs, than females mated 5 times (Gershman 2007). To avoid a bias in which crickets assigned to the lower mating frequency would be more likely to complete their assigned matings, individuals in each group were given equivalent periods of time in which to complete their assigned matings. Crickets from the 5 and 10 mating treatment groups were given 7 and 14 days, respectively, to complete their matings or they were dropped from the study.

Crickets were given one mating opportunity per night starting 5 days after eclosion. Mating trials took place daily at the start of the dark cycle using a 25-W red light. Crickets were housed individually in transparent 0.2-L cups with constant access to food (rabbit chow) and moist cheesecloth for water and oviposition. Each cricket was placed with a novel mate for 20 minutes in a clean 0.2 liter cup. Crickets were not presented with the same mate twice, because female crickets have been shown to prefer novel to previous mating partners (Bateman 1998; Ivy et al. 2005). Matings were counted as successful if a spermatophore was attached to the female’s genital opening. Crickets were removed from the mating arena and returned to individual housing cups 10 min after successful copulation to prevent additional matings. Individuals that completed their mating treatment before 14 days were transferred daily to an empty mating arena to allow for routine maintenance of the housing cup and to control for the handling stress experienced by the females still receiving mating opportunities.

At 20 days after adult eclosion, crickets were injected with 5 μL of 5 × 10⁴ cells/5 μL *S. marcescens*. Crickets were injected through the membrane beneath the pronotum with 5 μL of bacterial solution delivered by a 10-μL Hamilton syringe (Hamilton Co., Reno, NV). Preliminary tests indicated that the dosage of *S. marcescens* 5 × 10⁴ bacterial cells/5 μL solution killed approximately half of injected individuals. Bacterial solutions were made from freeze-dried *S. marcescens* (Carolina Biological Supply, Burlington, NC), which contained approximately 1 × 10⁵ bacterial cells per μl of broth. Bacterial counts were made using an Improved Neubauer Ultraline Spotlite counting chamber hemocytometer. The 5 × 10⁴ cells/5 μL solution was produced by diluting 100 μL of bacterial broth in 990 μL of insect saline (121 mM sodium chloride, 4.1 mM calcium chloride, 1.37 mM dibasic potassium phosphate, 198 μM monobasic potassium phosphate, and 23.6 mM Tris-hydrochloride, pH 7.4). Crickets were inspected daily for 7 days, and date of death was recorded for each individual. Most crickets that die from *S. marcescens* infection do so within 4 days of injection (Adamo et al. 2001). After death, the pronotum width of each individual was measured.

Of the 194 virgin females initially recruited into this experiment, 150 survived to day 15 and completed their matings. Seventy-two females completed the 5-mating treatment, and 78 females completed the 10-mating treatment. Of the 200 virgin males initially recruited into this experiment, 149 survived to day 15 and completed their assigned matings. Seventy-seven males completed the 5-mating treatment, and 72 males completed the 10-mating treatment.

**Experiment Ib: PO and lysozyme-like enzyme activity, and encapsulation ability**. One hundred and sixteen virgin males and 135 virgin females were randomly assigned to mate either 5 or 10 times using the same methods as described above. Of the 116 virgin males recruited, 52 males successfully completed the 5-mating treatment and 36 completed the 10-mating treatment. Of the 135 females, 48 females successfully completed the 5-mating treatment and 55 completed the 10-mating treatment.

Twenty days after eclosion, crickets were implanted with a 3-mm long sandpaper-roughened segment of 0.25-mm diameter nylon monofilament fishing line. A small hole was made ventrally between the fifth and sixth abdominal segments with a 27-gauge syringe needle, and the implant was inserted until it was completely within the cricket’s abdominal cavity. Prior to implantation, crickets were cold anesthetized for 10 min at 6 °C in a refrigerator, and implants and needles were sterilized in 70% ethanol. These procedures are similar to those used in other studies (Siva-Jothy et al. 1998; Rantala et al. 2000; Doums et al. 2002; Fedorka et al. 2004; Zuk et al. 2004). More recent studies have both questioned (Leclerc et al. 2006) and provided support (Rantala and Roff 2007) for the relationship between implant encapsulation to resistance to natural pathogens. After implantation, crickets were returned to their housing cups in the growth chamber and allowed to resume normal activity. A pilot study of 10 crickets demonstrated that 24 h was the most appropriate duration for the implants to remain in the crickets to provide a range of variation in melanization. Exactly 24 h after each cricket was implanted, hemolymph samples were drawn and crickets were placed in a 80 °C freezer. Three milliliters of hemolymph were mixed with 40 mL phosphate buffered saline (PBS) solution and frozen in the −80 °C freezer to induce cell lysis and to prevent enzymatic reactions from proceeding. Hemolymph samples were stored at −80 °C for at least 1 week prior to analysis.

Implants were dissected out of the frozen crickets and any clumps of tissue removed. Each implant was photographed using a Spot RT Color digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) mounted on a Leica stereomicroscope. Implants were photographed on a glass slide using light transmitted through the stage of the microscope. Encapsulation was measured as the darkness of each implant using the image analysis software Object-Image. (Object-Image, by N. Vischer, is an extended version of NIH Image, by W. Rasband, and is available at http://simon.bio.uva.nl/object-image.html). An automated program outlined each implant and provided the average grayscale value of all the pixels within each implant image. Darkness scores ranged from 0 to 255, with higher values indicating a greater degree of
darkness and encapsulation. Background gray scale was periodically checked for consistency by measuring the darkness of an implant-sized segment of empty slide. Twenty implants were measured twice to assess repeatability ($r$ using calculations described by Lessells and Boag, 1987), and implant darkness was found to be highly repeatable ($r = 0.90$, analysis of variance [ANOVA] $F_{9,99} = 18.65$, $P < 0.0001$).

To measure PO activity of hemolymph samples drawn from experimental individuals, a known quantity of L-DOPA (Acrors Organics, Geel, Belgium) was added to hemolymph to replace the naturally occurring substrate. Because the amount of L-DOPA is not a limiting factor in the experimental reaction leading to melanization, and was constant across samples, the subsequent melanization of the hemolymph must be due to variation in the amount of PO present in the hemolymph of individuals. Preliminary tests indicated that 2.9 mg L-DOPA/1 mL PBS buffer added to the hemolymph solution was sufficient to cause a reaction that leveled off within 3 h. To quantify melanization, 10 μL of the thawed frozen hemolymph sample along with 90 μL of PBS buffer with L-DOPA was added to each well of the spectrophotometer microplate and recorded at 490 nm using a Bio Rad 550 Microplate reader (Bio-Rad, Hercules, CA). This method estimates the total change in optical density (OD) over the course of the reaction, ranging from an OD of 0.000 (transparent) to 3.500 (opaque). OD readings were taken every 30 min for 3 h. Preliminary tests indicated that this range of readings produced a measurable and consistent change in OD over time. The PO activity rate was calculated as the change in OD over time.

To estimate lytic activity, 3 mg of Micrococcus lyodeikticus (ICN Biomedicals, Inc., Costa Mesa, CA) per liter of PBS buffer was added to hemolymph to determine the ability of a lysozyme to lyse the bacterial cells. Preliminary tests showed that this amount of Micrococcus caused visible cell lysis and activity to level off after 3 h. To quantify lytic activity, 10 μL of the thawed, frozen hemolymph sample along with 90 μL of PBS buffer containing L-DOPA were added to each well of the spectrophotometer microplate well and changes in OD recorded. This method estimates the total change in OD from opaque to clear as a lysozyme lyses the bacterial cells. OD readings were taken every 10 min for 2 h. Preliminary tests indicated that this range of readings produced a measurable and consistent change in OD over time. Although OD decreases as more bacterial cells are lysed, lytic activity is portrayed as a positive number for clarity. This experimental design does not permit the characterization of the specific lysozyme responsible for the cell lysis (Schneider 1985); the observed lytic activity is thus attributed to a lysozyme-like enzyme.

Data from 1 cricket were dropped from the lysozyme-like enzyme assay and 8 from the PO assay because the OD measurements for these samples were nonlinear, suggesting contamination or equipment malfunction. The 3 most extreme outliers, as determined by the outlier boxplot method in the JMP 5.1 statistical package (www.jmp.com), were dropped from further analysis, after which the data were normally distributed and homoschedastic. Controls were performed to test for differences in OD values across sample plates as previously described. Control samples did not differ significantly among sample plates (lysozyme: ANOVA $F_{2,47} = 0.271$, $P = 0.846$; PO: ANOVA $F_{2,161} = 0.317$, $P = 0.729$). Because pronotum width was not correlated with lysozyme-like enzyme activity ($F_{1,112} = 2.51$, $P = 0.116$), PO OD ($F_{1,108} = 0.0391$, $P = 0.844$), or implant darkness ($F_{1,113} = 0.785$, $P = 0.378$), it was dropped from further analyses.

## RESULTS

The effect of experimentally assigned matings on immune responses

### Experiment Ia: Experimental infection with S. marcescens

Logit regression was used to predict the probability that an individual would survive more than 4 days following bacterial infection, as influenced by the assigned number of matings, sex, and body size (pronotum width). Sex was a significant predictor of survival: females were more likely to survive experimental infection than males (Figure 1, Wald $\chi^2_{1} = 6.08$, $P = 0.014$). However, neither number of matings nor body size was significant predictor of survival (Figure 1, Wald $\chi^2_{1} = 0.49$, $P = 0.48$; Wald $\chi^2_{1} = 0.47$, $P = 0.49$).

![Figure 1](https://academic.oup.com/beheco/article-abstract/19/4/810/202850/13 December 2018)

The proportion of crickets that survived beyond 4 days after experimental infection with *Serratia marcescens*. Sample sizes for males and females that mated 5 and 10 times are 77, 72, 72, and 76.
Experiment Ib: PO and lysozyme-like enzyme activity and encapsulation ability

Multivariate analysis of covariance (MANCOVA) was employed to determine the effect of number of matings, sex, and body size on the 3 assays of immunity. MANCOVA allows analysis of multiple dependent variables while controlling for pseudoreplication error associated with multiple use of the same individuals. Because body size did not have a statistically significant effect on immunity, body size was dropped from the final model (Table 1a). Number of matings did not have a significant effect on immunity, nor was there any significant interaction between sex and numbers of matings (Table 1a). Sex had a significant effect on immunity, with females having stronger immune responses than males (Table 1a, Figure 2). Although females had significantly higher PO activity and were better at melanizing implants, males and females did not differ in their ability to lyse bacterial cells (Table 1b, Figure 2). Further, univariate ANOVAs on female lysozyme-like enzyme activity indicated that although females mated 5 times did not differ from males (ANOVA \( F_{1,112} = 0.306, P = 0.58 \)), females that mated 10 times had lower immunity than females that mated 5 times (ANOVA \( F_{1,86} = 9.19, P = 0.003 \)). Number of matings did not affect female PO (\( F_{1,86} = 2.64, P = 0.11 \)) or implant darkness (\( F_{1,86} = 0.76, P = 0.39 \)).

### Table 1

(a) Multivariate analysis of covariance of the effect of body size, sex, number of assigned matings, and the interaction between number of matings and sex on lysozyme-like enzyme activity, PO activity, and implant darkness and (b) ANOVAs of the effect of sex on lysozyme-like enzyme activity, PO activity, and implant darkness for assigned matings

<table>
<thead>
<tr>
<th>(a) MANCOVA</th>
<th>df</th>
<th>Wilks’ ( \lambda )</th>
<th>( F )</th>
<th>( P )</th>
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<tr>
<td>Body size (covariate)</td>
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<td>0.798</td>
<td>0.497</td>
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<tr>
<td>Sex</td>
<td>3,145</td>
<td>7.40</td>
<td>0.000*</td>
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<tr>
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<td>0.172</td>
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<tr>
<td>Number of matings × sex</td>
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<td>1.71</td>
<td>0.168</td>
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<tr>
<td>(b) Sex</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Lysozyme</td>
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<td>3.026</td>
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<td></td>
</tr>
<tr>
<td>PO</td>
<td>1,150</td>
<td>10.147</td>
<td>0.002*</td>
<td></td>
</tr>
<tr>
<td>Implant darkness</td>
<td>1,150</td>
<td>11.195</td>
<td>0.001*</td>
<td></td>
</tr>
</tbody>
</table>

\( P \) values indicated by an asterisk are statistically significant with a sequential Bonferroni correction. \( df = \) degrees of freedom.

Experiment IIb: PO and lysozyme-like enzyme activity and encapsulation ability

Multivariate analysis of covariance (MANCOVA) was employed to determine the effect of number of matings, sex, and body size on the 3 assays of immunity. MANCOVA allows analysis of multiple dependent variables while controlling for pseudoreplication error associated with multiple use of the same individuals. Because body size did not have a statistically significant effect on immunity, body size was dropped from the final model (Table 1a). Number of matings did not have a significant effect on immunity, nor was there any significant interaction between sex and numbers of matings (Table 1a). Sex had a significant effect on immunity, with females having stronger immune responses than males (Table 1a, Figure 2). Although females had significantly higher PO activity and were better at melanizing implants, males and females did not differ in their ability to lyse bacterial cells (Table 1b, Figure 2). Further, univariate ANOVAs on female lysozyme-like enzyme activity indicated that although females mated 5 times did not differ from males (ANOVA \( F_{1,112} = 0.306, P = 0.58 \)), females that mated 10 times had lower immunity than females that mated 5 times (ANOVA \( F_{1,86} = 9.19, P = 0.003 \)). Number of matings did not affect female PO (\( F_{1,86} = 2.64, P = 0.11 \)) or implant darkness (\( F_{1,86} = 0.76, P = 0.39 \)).

DISCUSSION

In the present study, females were consistently superior to males in immunity. Females were better in both “realized” immune response, the response of individuals to a novel immune challenge (implant encapsulation and \( S. \) marcescens infection), as well as their “potential” immune response, the potential of an individual to mount an immune response (PO activity). Clearly, immunity does not exist as a single trait but is the result of many independent and interacting physiological responses; the assays used in this study represent a small subset of possible immune responses. PO activity and implant darkness are both related to melanin production and may show

**Figure 2**

The effect of number of assigned matings and sex on (a) change in lysozyme-like enzyme assay OD, (b) change in phenoloxidase assay OD, and (c) implant darkness. Means, standard errors, and sample size of each treatment are shown.

The effect of ad libitum mating on immune responses

Experiment II. PO and lysozyme-like enzyme activity and encapsulation ability

Multivariate analysis of covariance was employed to determine the effect of number of matings, sex, and pronotum width (covariate) on the change in lysozyme-like enzyme OD, change in PO OD, and implant darkness. Neither number of matings, nor the interaction between sex and number of matings, had an effect on immunity (Table 2a). Sex had an effect on immunity, with females having stronger immune responses than males (Table 2a, Figure 3). In univariate ANOVAs of the effect of sex on the different immune assays, females had higher immunity than males in PO activity but not in lysozyme-like enzyme activity or implant darkness (Table 2b, Figure 3). Unlike experiments in which females were preassigned a prescribed number of matings, the number of times that females mated was not correlated with lysozyme-like enzyme activity \( (R^2 = 0.003, F_{1,53} = 1.87, P = 0.18) \).
physiologically nonindependent responses. However, melanin production is not expected to be physiologically related to either lysozyme activity, which usually acts on Gram-positive bacteria (Schneider 1985) or infection by the Gram-negative bacterium *S. marcescens*. The complex nature of immunity can lead to trade-offs between components of immunity (Adamo 2004; Rantala and Roff 2005). However, that females had superior immunity to males across multiple measures of immunity and different experimental designs is an unexpectedly robust result, suggesting that strong selective pressure may cause multiple independent immune traits to respond in the same way. Although epistasis and sex linkage may explain sex differences in immunity that are independent from life history–based theory (Rantala and Roff 2007), these gene interactions may also contribute to congruence in results among measures of immunity. The results of this study provide support for the hypothesis that differences in life history among males and females may lead to differences in immunity (Zuk 1990).

For most immune measures, with one exception, the number of matings had no effect on the strength of immune responses. However, previous studies have shown that mated individuals have lower immunocompetence than virgin individuals (Adamo et al. 2001; Rolff and Siva-Jothy 2002; for an exception, see Shoemaker et al. 2006). Taken together, these findings suggest that although mating may result in an initial cost to immunity, multiple matings beyond the first mating do not add appreciably to this cost (for an exception, see Baer et al. 2006). The absence of cumulative costs of multiple mating may allow females to reap even modest benefits of supernumerary matings and partially account for the pervasiveness of multiple mating and polyandry in female insects.

Females that were assigned to mate more times had reduced lysozyme-like enzyme levels, suggesting that multiple mating can harm this component of female immunity. Mating did not affect other immune responses, including bacterial challenge, which Adamo (2004) found to be correlated with lysozyme-like enzyme levels. It is possible that a subtle difference in lysozyme-like enzyme activity could not be detected by this bacterial assay. Alternatively, the effect of lysozymes on gram-negative bacteria may be mediated by other factors. Given the complexity of immunity, mating has the potential to affect specific immune reactions, as well as influence interactions between components of immunity (McGraw et al. 2004).

In field crickets, males have extremely complex accessory glands (Kaulenas 1992) that produce chemical compounds known to stimulate female egg production and reduce female sexual receptivity (Loher and Dambach 1989). Fedorka and Zuk (2005) describe a possible chemical pathway by which male *Allonemobious* cricket seminal contributions could lead to reduced female PO activity. Ejaculatory substances transferred from males to females at mating could potentially cause the
observed decrease in female lysozyme-like enzyme levels with increasing numbers of matings. However, when females were allowed to mate ad libitum, females that mated more times did not exhibit reduced immunity. It is possible that individuals in superior condition are capable of mating at a high rate without an immunity trade-off (Rantala and Kortet 2003; Rantala et al. 2005). Alternatively, when females are free to choose how many times to mate, they may differentially moderate their mating frequency so as to avoid costs to their immune system.

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